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Identical Carbonic Anhydrase Contributes to Nacreous or Prismatic Layer Formation in *Pinctada fucata* (Mollusca: Bivalvia)

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Abstract. We have found a carbonic anhydrase (CA) in the prismatic layer of *Pinctada fucata.* This CA has the same kinetic properties as Nacrein, which is a CA existing in the nacreous layer of *Pinctada fucata.* We have examined the effects of inhibitors on the enzyme activity. Sodium sulfide and sulfanilamide are typical inhibitors of various types of CA; however, a CA in the prismatic layer and Nacrein were found to be resistant to sodium sulfide and to show a weak resistance to sulfanilamide. This is the first report of a carbonic anhydrase with resistance to sodium sulfide. The molecular mass of the prismatic layer CA was estimated by SDS-PAGE to be approximately 60 kDa. Moreover, we have determined the N-terminal amino acid sequence of a CA in the prismatic layer. The sequence of the first 11 amino acids was in agreement with that of Nacrein, as deduced from the cDNA sequence. From these results, we have concluded that the carbonic anhydrase of the prismatic layer is Nacrein. Nacrein contributes to the formation of a prismatic layer as well as a nacreous layer of mollusk shells as a carbonic anhydrase and is a matrix component.

INTRODUCTION

Calcite, aragonite, and vaterite are crystal polymorphisms of calcium carbonate in biomineralization (Lowenstam, 1981; Lowenstam & Weiner, 1989). Of these, calcite is the most stable, and vaterite is the most unstable. Aragonite is slightly less stable than calcite at ambient temperature, but is widespread in marine organisms. Mollusk shells are composed of aragonite and/or calcite, and the organic matrix comprises 0.01–5% by weight of the shells. In the case of the pearl oyster *Pinctada fucata* (Gould, 1850), the outer prismatic layer contains calcite, and the inner nacreous layer contains aragonite. These layers contain organic matrix secreted by the mantle epithelia. The organic matrix consists of EDTA-soluble and insoluble proteins (Hare, 1963; Watabe, 1984; Mann, 1988). The formation of the two types of crystal is regulated by the matrix protein constituents. Some of them play an important role in the chemical control of crystal polymorphisms (Belcher et al., 1996; Falini et al., 1996; Samata et al., 1999).

It has been suggested that carbonic anhydrases (CA) that catalyze the interconversion of $CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$ participate in the process of calcification (Benesch, 1984; Kakei & Nakahara, 1996) and mollusk shell formation (Wilbur & Jodrey, 1955; Freeman, 1960; Medakovic & Lucu, 1994). It is believed that the CA of mantle epithelium facilitates the secretion of HCO_3^- for this calcification (Boer & Witteveen, 1980). We have already shown that a 60 kDa protein called Nacrein, which exhibits CA activity, exists in the EDTA-extract of the nacreous layer of oyster pearls (Miyamoto et al., 1996). Nacrein is an important factor in calcium carbonate crystallization, acting as a structural protein and a catalyst that provides the carbonate ion. We predicted that a Nacrein-like protein also participates in formation of the prismatic layer. Based on the results of the present study, we have now identified and characterized a CA in the EDTA-extract of the prismatic layer of *Pinctada fucata.* Here we report the biochemical properties of prismatic layer CA and discuss the function of CA in biomineralization.

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MATERIALS AND METHODS

Isolation of EDTA-Soluble Proteins

The prismatic layer was separated by cutting the shell edges with scissors. After the removal of epiphytes, the shell was crushed to a fine powder. The powdered shell (20 g) was extracted with 100 mL of 0.5 M EDTA (pH 8.0) with continuous stirring for 3 days at room temperature. The EDTA-soluble fraction was isolated from the insoluble matrix by centrifugation at ×30,000 g for 20 min. The supernatant (80 mL) was dialyzed against 3 liters of H_2O with three changes. The dialyzed fraction (300 mL) was lyophilized and then dissolved in 10 mL of 10 mM Tris-HCl (pH 8.0). The sample was dialyzed against 10 mM Tris-HCl (pH 8.0), followed by concentrated. Preparation of the EDTA-soluble extract of the nacreous layer of *Pinctada fucata* is the same as de-

scribed above. The amount of protein was determined by using Protein Assay Dye Reagent (Bio-Rad).

Carbonic Anhydrase (CA) Assay

The assay of carbonic anhydrase activity was performed as described by Miyamoto et al. (1996). Six drops of phenol red, 3 mL of 20 mM Veronal buffer (pH 8.3), and 20–200 μL of the test-material-containing solution were mixed and placed in ice water. The reaction was started by the addition of 2 mL of ice-cold water saturated with CO_2, and then the time for the pH to drop to 7.3 was measured. Definition of units is as follows: unit = $(T_0 - T)/T$, where T and T_0 are the reaction times required for the pH change from 8.3 to 7.3 at 0°C with and without a catalyst, respectively. Assay of enzyme activity in the presence of inhibitor was carried out as follows: all reagents in the assay mixture except the substrate were premixed in the reaction vessel for 10 min at 0°C. The reaction was started by the addition of the substrate.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were subjected to sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis on 10% acrylamide gels, as described by Laemmli (1970).

N-Terminal Amino Acid Determination

Proteins of the EDTA-soluble fraction were separated by 10% SDS-PAGE and were blotted onto a PVDF membrane (Millipore) using a dry blotting system (Nippon Eido). After Ponceau S staining, the band was cut out and then subjected to N-terminal amino acids sequence analysis.

DEAE-Sephacel Column Chromatography

Approximately 20 mg of the soluble fraction of the prismatic layer was loaded onto a DEAE-Sephacel (Pharmacia) ion-exchange column (10 × 1.5 cm) equilibrated with 10 mM Tris-HCl (pH 8.0). After washing the column with 5 mL of 10 mM Tris-HCl (pH 8.0), the soluble fraction was chromatographed in the same buffer at a flow rate of 8 mL/h using a liner 0–0.8 M NaCl gradient. Fractions of 2 mL were collected, and carbonic anhydrase activity was assayed. The fractions containing carbonic anhydrase (Fraction Number 31–40) were pooled and then dialyzed for 10 mM Tris–HCl (pH 8.0), followed by concentration to 1 mL.

Gel Filtration Chromatography

The concentrated sample was chromatographed over a Cellulofine GCL-300 sf (Seikagaku Kogo Co.) column (95 × 1.5 cm) equilibrated with 10 mM Tris-HCl (pH 8.0) containing 0.2 M NaCl at a flow rate of 12 mL/hr.

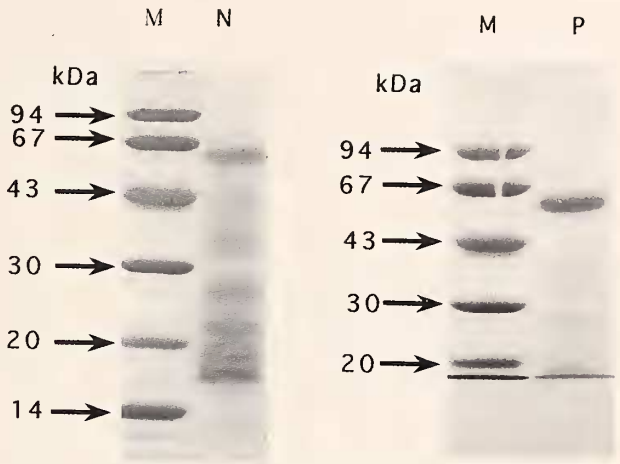

Figure 1. Comparison of SDS-PAGE Pattern of EDTA-soluble proteins extracted from the nacreous and prismatic layers of *Pinctada fucata*. 10 μg of proteins were subjected to 10% SDS-PAGE. Lane N, EDTA-soluble proteins were extracted from the pearl nacreous layer. Lane P, EDTA-soluble proteins were extracted from the prismatic layer. Lane M, Protein markers (GIBCO BRL).

Fractions of 1.2 mL were collected, and carbonic anhydrase activity was assayed. Each fraction containing carbonic anhydrase was dialyzed separately for 10 mM Tris-HCl (8.0) followed by concentration to approximately 100 μL. To examine the purity, 20 μL of the concentrated fractions was subjected to 10% SDS-PAGE.

RESULTS

The protein components of the EDTA-soluble fraction in the prismatic layer were compared with those of the nacreous layer. Proteins were loaded onto 10% SDS-PAGE. The molecular mass of the major protein was approximately 60 kDa in both layers (Figure 1). The 60 kDa protein of the nacreous layer is Nacrein (Miyamoto et al., 1996).

We assayed the carbonic anhydrase (CA) activity in the soluble fraction extracted from the prismatic layer. Bovine erythrocyte CA and Nacrein in the soluble fraction of the nacreous layer showed notable CA activity (Table 1). The soluble fraction extracted from the prismatic layer also contained CA activity. Although the specific activity was relatively lower than that of bovine erythrocyte CA, it was almost the same as that of Nacrein. The velocity of the enzyme reaction was increased 2 times by using a twofold amount of enzyme. A large amount of Bovine Serum Albumin (BSA), which has no enzyme activity, and a heat-inactivated soluble fraction showed no CA activity. These results indicate the presence of carbonic anhydrase in the soluble fraction extracted from the prismatic layer of *Pinctada fucata*.

We next examined the effects of inhibitors on the CA activity. Sodium sulfide and sulfanilamide are typical in-

Table 1

Assay of carbonic anhydrase activity of the EDTA-soluble fraction extracted from the prismatic layer of *Pinctada fucata*.

Sample	Amount used for assay (mg)	T (sec)	Specific activity (units/mg)
—	—	410 (= T_0)	—
BSA[1]	6	430	—
BECA[2]	0.3	50	2.4×10^4
Soluble fraction (nacreous layer) (Nacrein)	3	40	3.3×10^3
Soluble fraction (prismatic layer)	1.5	100	2.1×10^3
Soluble fraction (prismatic layer)	3	50	2.1×10^3
Soluble fraction (prismatic layer) (heat-inactivated)	3	400	—

[1] BSA: Bovine serum albumin.
[2] BECA: Bovine erythrocyte carbonic anhydrase.

hibitors of various types of carbonic anhydrases (Val, 1996). The activity of bovine erythrocyte CA was almost inhibited by these inhibitors (Table 2), as reported previously (Kiese & Hasting, 1940; Davenport, 1945). However, Nacrein was resistant to sodium sulfide and showed weak resistance to sulfanilamide. These results were identical to those for the prismatic layer CA.

To purify the carbonic anhydrase in the prismatic layer, we performed column chromatography. After DEAE-Sephacel column chromatography, the concentrated sample was passed through a Cellulofine GCL-300 sf column (Figure 2). Each fraction containing an enzyme activity was dialyzed separately for 10 mM Tris-HCl (8.0) and then concentrated to approximately 100 μL. To examine the purity, the concentrated fractions were applied to 10% SDS-PAGE. Fraction number 35 showed a single protein band that was almost homogeneous (Figure 3). The protein had an approximate molecular mass of 60 kDa. Fraction number 39 contained a larger amount of the 60 kDa protein than that of fraction 35 as well as a large amount of contamination which had an approximate molecular mass of 40 kDa. However, the total enzyme activity of this fraction was approximately 1.7 times greater than that of fraction 35. From these results, we conclude that the approximate molecular mass of the prismatic layer CA is 60 kDa.

We have determined the N-terminal amino acid sequence of the 60 kDA protein. The sequence of the first 11 amino acids agreed with that of Nacrein as deduced from the cDNA sequence (Figure 4).

DISCUSSION

Carbonic anhydrase (CA) is a ubiquitous enzyme existing in every tissue and cell type. Various isozymes of CA are now known (Tashian, 1989; Henry, 1996) and play an important role in acid-base balance, ion transport, maintenance of ionic concentration, and modulation of hemoglobin O_2 affinity (Cameron, 1979; Henry, 1984; Forster et al., 1986). CA also participates in biomineralization, and it is well known that CA is an essential enzyme of calcification (Wilbur & Jodrey, 1955; Freeman, 1960;

Table 2

Comparison of the effects of inhibitors on the activity of carbonic anhydrases. CA activity was expressed as a percentage of the activity in the absence of inhibitor.

Sample	Used amount for assay (mg)	Relative activity inhibitor		
		—	Sodium sulfide	Sulfanilamide
BECA*	1	100		
	1		11	
	1			2
Nacreous layer soluble fraction (Nacrein)	1.5	100		
	1.5		100	
	1.5			48
Prismatic layer soluble fraction	1.5	100		
	1.5		100	
	1.5			40

* BECA: Bovine erythrocyte carbonic anhydrase.

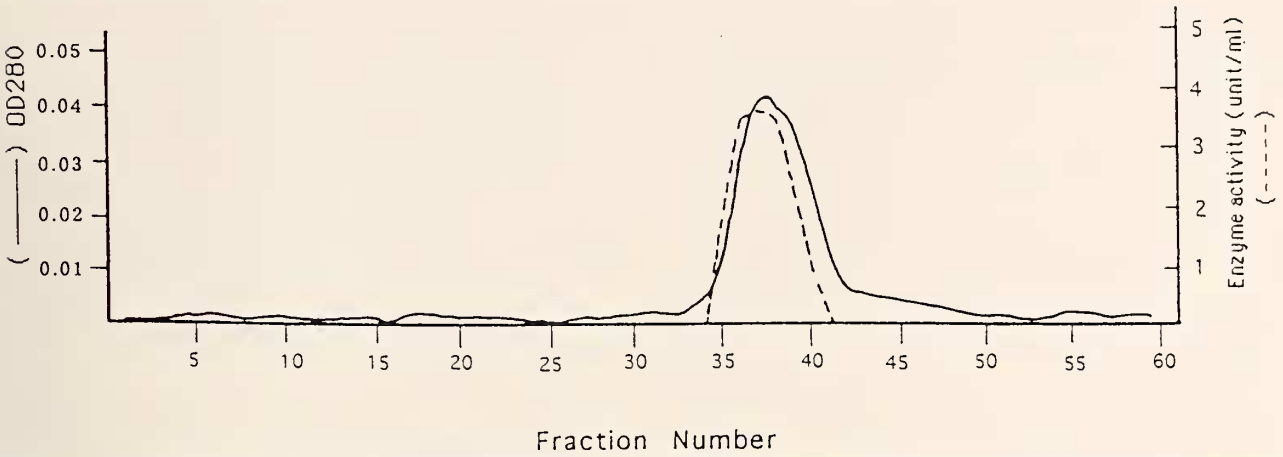

Figure 2. Cellulofine GCL-300 sf chromatographic profile of CA-containing fractions obtained from a DEAE-Sephacel column chromatography. Solid line, absorbance at 280 nm; dashed line, enzyme activity.

Bore & Witteveen, 1980). The mantle of the mollusk shell contains CA activity (Medakovic & Lucu, 1994; Freeman & Wilbur, 1948). We have recently identified the carbonic anhydrase named Nacrein in the EDTA-soluble matrix of the nacreous layer in oyster pearls and have isolated its cDNA (Miyamoto et al., 1996). Based on the amino acid sequence, Nacrein appears to contain two functional domains, one a carbonic anhydrase domain and the other a Gly-Xaa-Asn (Xaa = Asp, Asn, or Glu) repeat domain. It has been assumed that Nacrein contributes to the formation of HCO_3^- ions in calcification, and functions as a matrix component of aragonite crystal. The prismatic layer contains calcite, which is another polymorphism of $CaCO_3$, in addition to aragonite of the nacreous layer. We therefore assumed that the prismatic layer contains a carbonic anhydrase that differs from Nacrein, and that this enzyme contributes to calcite formation.

We have identified in the present study a carbonic anhydrase in the extract of the prismatic layer of *Pinctada fucta*. The specific activity was found to be relatively lower than that of bovine erythrocyte CA and almost the same as that of Nacrein.

Sodium sulfide and sulfanilamide are well known inhibitors of CAII (Davis, 1959, 1961). The carbonic anhydrase in the prismatic layer was found to be resistant to sodium sulfides and to have weak resistance to sulfanilamide. These results are almost the same as those for Nacrein. This is the first report, however, of a carbonic anhydrase resistant to sodium sulfide. The mechanism of resistance to sodium sulfide is unknown.

To determine the molecular mass of carbonic anhydrase in the prismatic layer we further purified the protein

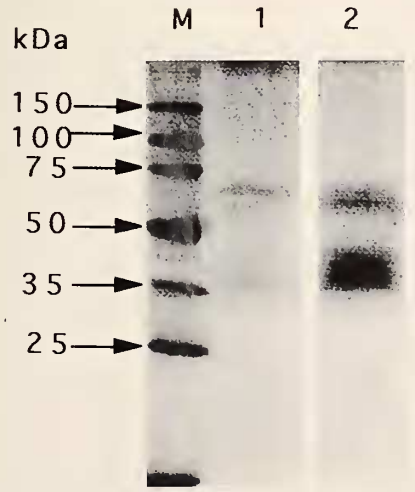

Figure 3. SDS-PAGE electrophoretic pattern of CA-containing fractions. Lane M, Perfect Protein® Markers (Novagen). Lanes 1 and 2 correspond to the fraction numbers of 35 and 39, respectively.

A

1	2	3	4	5	6	7	8	9	10	11
A	(S)	M	F	K	H	D	H	Y	M	D

B

1	2	3	4	5	6	7	8	9	10	11
A	S	M	F	K	H	D	H	Y	M	D

Figure 4. Sequence alignment of the N-teminal of the prismatic layer CA with Nacreion. A. Prismatic layer CA. B. Nacrein. The amino acid in parentheses is uncertain.